

Investigating the Effect of CoCl_2 Administration on Diabetic Nephropathy and Associated Aortic Dysfunction

A.B. Deshmukh^{a, b} J.K. Patel^c A.R. Prajapati^e Bharat Mishra^d

^aDepartment of Pharmacology, Shankersinh Vaghela Bapu Institute of Pharmacy, Unava, ^bDepartment of Pharmaceutical Science, Bhagwant University, Ajmer, Departments of ^cPharmaceutics and ^dPharmacology, Nootan Pharmacy College, Visnagar, and ^eDepartment of Quality Assurance, Shree Sarvajani Pharmacy College, Mehsana, India

Key Words

Chronic hypoxia • Hypoxia inducible factor-1 α • Prolyl hydroxylase • Oxidative stress • Nitric oxide • Angiotensin II • Endothelial dysfunction

Abstract

Aim: Endothelial dysfunction appears to be a consistent finding in diabetic nephropathy. The study aimed to investigate the effect of cobalt chloride in the amelioration of endothelial dysfunction in uninephrectomized diabetic rats.

Methods: We examined the effect of CoCl_2 (10 mg/kg, i.p., OD = once a day) treatment on contractile responses to angiotensin II (10^{-10} to 10^{-6} M) in an aortic preparation of control rats and uninephrectomized diabetic control rats. Blood glucose, plasma urea, creatinine, uric acid, aortic endothelial nitric oxide synthase (eNOS), nitrate/nitrite (NOx), superoxide dismutase, catalase and reduced glutathione levels were checked in the different groups. **Results:** A significant attenuation of the augmented responses to angiotensin II was observed in CoCl_2 -treated animals along with a fall in plasma urea, creatinine and uric acid levels. A significant reduction in blood glucose and an increase in aortic eNOS and NOx levels along with antioxidants levels were observed. **Conclu-**

sion: Chronic hypoxia augments angiotensin II responses in the thoracic aorta of uninephrectomized diabetic control rats. CoCl_2 attenuates these enhanced vascular responses with a significant decrease in blood glucose signifying stabilization of the hypoxia-inducible factor in the alleviation of endothelial dysfunction in diabetic nephropathy.

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Introduction

Compelling evidence shows that hyperglycemia and hypoxia may play an essential pathophysiological role in the complications of diabetes resulting from a defective response of the tissues to low oxygen tension [1]. Recent data show that angiotensin II produces tissue hypoxia [2] and these findings suggest a link between cellular hypoxia and AT_1 receptor upregulation in angiotensin II-induced endothelial injury. Hypoxia-inducible factors (HIFs) comprise the family of transcription factors which exert regulatory mechanisms by influencing gene expression during hypoxia; this transactivates in the nucleus a host of genes and exerts physiological effects [3]. Captivatingly, cobalt enhances HIF activity by inhibiting HIF

degradation by prolyl 4-hydroxylases [4]. The study was designed to explore the role of cobalt chloride-induced augmentation of HIF activation in the amelioration of diabetic nephropathy and the associated vascular dysfunction.

Design and Methods

Animals

Sprague-Dawley rats, 8 weeks of age, procured from the Torrent Research Centre, Gujarat, India were maintained in well-controlled humidity (<70%) and temperature (<30°C) with a 12-hour day and night cycle at the Central Animal Facility, Nootan Pharmacy College, India (CPCSEA Reg. No. 1244/ac/08/CPCSEA). Each rat had free access to untreated tap water and standard rat chow (Pranav Agro Ltd., Ahmedabad, India) according to the norms of the Institutional Animal Ethics Committee and CPCSEA.

Induction of Diabetic Nephropathy

The rats had their right kidney removed to accelerate the development of diabetic nephropathy as described previously [5]. One week after surgery, a single intraperitoneal dose of streptozotocin (STZ; Future Bioscience Pvt. Ltd., Delhi, India; 40 mg/ml in 0.1 mol/l phosphate/0.4 mol/l citrate buffer, pH 6.5) at a dose of 45 mg/kg body weight (selection of dose after standardization) was injected in uninephrectomized animals [6]. The diagnosis of diabetes was established 48 h after the STZ injection by the determination of the tail vein blood glucose and animals having a 4- to 6-hour fasting blood glucose concentration of less than 200 mg/dl were excluded from the study.

Grouping of Animals

The animals were grouped as follows: (1) control animals (n = 6), (2) animals with unilateral nephrectomy followed by STZ administration without treatment (n = 6; uninephrectomized diabetic), (3) animals with unilateral nephrectomy followed by STZ administration with CoCl₂ treatment (10 mg/kg, i.p.; n = 6; treatment group) and (4) control animals with CoCl₂ treatment (n = 6; control + CoCl₂).

Estimation of Parameters

Biochemical Parameters

The change in the body weight and food intake were recorded at basal and after 1, 3 and 5 weeks of study. Plasma samples were collected at basal and after 1, 3 and 5 weeks of study and were used for estimation of creatinine (Jaffé method), uric acid and urea (kinetic UV test) by semiautoanalyzer (Erba Mannheim) [7]. The glucose level (mg/dl) was estimated at different time points by commercially available glucose kits based on the glucose oxidase method (Horizon®, OneTouch, Johnson & Johnson, India).

Vascular Reactivity Study in Thoracic Aorta

Thoracic aorta spiral preparations of 20 mm length and 3 mm width were made by cutting the aorta causing no damage to the endothelium and were mounted in an organ bath (10 ml) containing Krebs-Henseleit buffer. The solution was continuously aerat-

ed with carbogen (95% O₂ + 5% CO₂) at 37°C. After recording two wake-up responses of KCl (80 mM) concentration-response curves to angiotensin II (Calbiochem, Darmstadt, Germany; 10⁻¹⁰ to 10⁻⁶ M) were recorded on a chart recorder (Bio-Device, Ambala, India) in the different groups using an isotonic transducer. The contractions measured in millimeters were expressed as mean ± SEM for the calculation of percent contraction, pD₂ values and E_{max}.

Estimation of Endothelial Nitric Oxide Synthase (pg/mg) and Nitrate/Nitrite Level (μmol/g) in Thoracic Aorta

The protein levels of endothelial nitric oxide synthase (eNOS) in thoracic aorta homogenate were determined using an ELISA kit (R&D Systems) according to the manufacturer's instructions and the tissue nitrate/nitrite (NOx) concentration was determined by using the Griess reaction [8] in Group 1, 2, 3 and 4.

Estimation of Superoxide Dismutase Activity (U/mg protein), Catalase Activity (U/mg protein) and Glutathione (mM/100 mg tissue)

Superoxide dismutase (SOD), catalase activity and glutathione (GSH) were measured in homogenized aortic supernatants by previously explained methods [9–11].

Cobalt Chloride Preparation and Treatment

Cobalt chloride hexahydrate solution (SD Fine Chemicals, Mumbai, India) was freshly prepared by using 200 mg of cobalt chloride in 20 ml normal saline to produce 10 mg/ml CoCl₂ solution. Animals were treated with cobalt chloride at the dose of 10 mg/kg, i.p., OD [12] for 30 days with continued dosing for 1 week followed by intermittent dosing at day 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 and 30. The animals in group 1 were administered with the equivalent amount of citrate buffer and normal saline (1 ml/kg) intraperitoneally and group 4 received citrate buffer and CoCl₂ treatment.

Statistical Analysis

Results were expressed as mean ± SEM. Comparisons were done on Graphpad, PRISM® 5 using one-way analysis of variance followed by Tukey's multiple comparison test. Data were considered statistically significant at p < 0.05 and highly significant at p < 0.001.

Result

Effect of CoCl₂ Treatment on General Features and Biochemical Parameters

Animals in group 2 showed a gradual decrease in their body weight with no significant change in groups 1 and 4. Food intake in groups 1 and 4 remained nearly constant throughout the 5-week period but a reduction of food intake was observed in group 2. A significant difference in food intake was observed in groups 2 and 3 (online suppl. table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000343888). A significant rise was observed in group 2 as compared to the control group

with a partial normalization in group 3 (online suppl. fig. 1A–C). Treatment with CoCl_2 (10 mg/kg, i.p.) significantly decreased the plasma glucose level (online suppl. fig. 3).

Attenuation of the Augmented Responses to Exogenous Angiotensin II by CoCl_2 in Aortic Preparations

The contractions to exogenous angiotensin II was significantly augmented in uninephrectomized diabetic aortic preparations compared to controls as was evident from the supersensitivity (increase in pD_2 value) and increased maximum response (E_{max}) (online suppl. table 2). A significant attenuation of vascular contractions to angiotensin II was observed in the CoCl_2 -treated group. The pD_2 and E_{max} values of angiotensin II were decreased with CoCl_2 treatment in the uninephrectomized diabetic group. CoCl_2 treatment attenuated the augmented vasoconstrictive responses to angiotensin II confirming the involvement of chronic hypoxia in the altered vascular reactivity to angiotensin II in diabetes (online suppl. fig. 2A–D).

Effect of CoCl_2 Treatment on eNOS and NOx Levels

A significant decrease of 45.54% in eNOS expression was observed in group 2 compared with the controls ($p < 0.001$). A significant increase of 39.35% was observed in the CoCl_2 -treated group as compared to the uninephrectomized diabetic aortic preparations ($p < 0.01$) (online suppl. fig. 4). A significant decrease was observed in tissue levels of group 2 (52.0 ± 3.0) which was significantly different from the control group ($p < 0.05$). However, a significant difference was observed in the tissue NOx levels of the treatment group (64.0 ± 2.2) as compared to group 2 (online suppl. fig. 5). Analysis of these data revealed that an association was observed in the levels of eNOS, NOx and the vascular function. Decreased levels of eNOS and NOx were observed in uninephrectomized diabetic rats and thus augmented responses were observed in angiotensin II-induced contraction as well. Treatment with CoCl_2 increased the levels of eNOS and NOx along with an attenuation of the augmented responses to angiotensin II. Thus eNOS-induced synthesis of NO and thus its contribution to the attenuation in the augmented responses were observed.

Effect of CoCl_2 Treatment on SOD Activity, Catalase Activity and GSH

A decreased level of aortic SOD in group 2 (4.3 ± 0.2) was found to be significantly elevated in group 3 ($10.6 \pm$

0.4). The tissue catalase level was found to be elevated in group 3 (61.0 ± 2.5) as compared to group 2 (37.0 ± 1.8). Group 3 animals showed a significant increase in aortic GSH levels (37.4 ± 1.6) which was significantly different from group 2 (21.4 ± 1.1) (online suppl. table 3).

Discussion

Unilateral nephrectomy followed by STZ administration-induced diabetes in the rat is a well-documented model of experimental diabetes [13]. Cobalt has been previously reported in renal injury animal models [14]. Conversely, a study demonstrated that treatment of STZ-induced diabetic rats with CoCl_2 results in a significant reduction in the serum glucose concentration [15]. Thus cobalt chloride treatment has immense possibilities for renal protection and reducing hyperglycemia in rats, which may establish HIF activation as well as prolyl 4-hydroxylase inhibitors as a newer therapeutic strategy for diabetic nephropathy. The results of this study demonstrated that uninephrectomized STZ diabetic nephropathy leads to a significant rise in plasma creatinine, uric acid and urea as compared to the control group. Reduction in body weight was observed in group 2 with no significant reduction in group 3; thus weight reduction may be attributed to the STZ effect and reduced food intake rather than the effect of CoCl_2 .

The contraction to exogenous angiotensin II was augmented in the aortic preparation of group 2 rather than the control group, which may be due to a modulation of vascular tone and structure. The increase in the E_{max} and pD_2 values of angiotensin II in uninephrectomized diabetic aortic preparations as compared to controls shows hyperreactivity of the vascular system due to endothelial dysfunction. These augmented responses to angiotensin II were attenuated in group 3 with a decrease in the pD_2 and E_{max} values. The vascular responses to angiotensin II in group 3 were restored to near control values with no significant differences observed compared to group 1. These findings strongly support the interpretation that chronic hypoxia may be the mediator of these augmented contractions and the observed attenuation of contractions shows that CoCl_2 exerts its effect by a regulation of gene expression of proteins, i.e. eNOS and thus NOx synthesis may be boosted with increased availability of eNOS which was further confirmed by ELISA. A corresponding increase in the levels of aortic tissue NOx was also observed, which confirmed that CoCl_2 increased NOx levels in vessels and was responsible for the attenuation of

the angiotensin II responses along with an increase in antioxidant activities. The present data demonstrate that cobalt chloride mitigates the development of endothelial dysfunction induced due to chronic hypoxia as well as hyperglycemia as evidenced by decreased blood glucose. eNOS and NOx, the products of HIF-regulated gene ex-

pression, were found to be augmented, which exerted a beneficial effect on the vascular functioning. In conclusion, our observations confirm that cobalt treatment improves endothelial functioning by upregulation of hypoxia-related protein content as well as the inhibition of oxidative stress in diabetic nephropathy.

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